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Immunological effects of cerebral palsy and rehabilitation exercises in children

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ABSTRACT

Cerebral palsy (CP) is a group of motor disorders caused by non-progressive lesions of the premature brain with lifelong pathophysiological consequences that include dysregulation of innate immunity. Persistent inflammation with increased levels of circulating pro-inflammatory tumor necrosis factor alpha (TNF-a) is negatively associated with rehabilitation outcome in children with CP. Because of the crosstalk between innate and adaptive immunity, we investigated the effect of CP and rehabilitation exercises on the adaptive immune system in children with CP by measuring the levels of $CD3^+$, $CD4^+$, $CD8^+$ T-cells, and $CD22^+$ B-cells and the levels of immunoglobulins. Children with CP had higher levels of $CD3^+$, $CD4^+$, $CD8^+$ T-cells, and $CD22^+$ B-cells compared to healthy children, and the rehabilitation exercise programs produced better outcomes in terms of increased gains in motor function at an earlier age. Rehabilitation exercises performed over a month resulted in significantly decreased levels of IgA in serum and reduced numbers of B-lymphocytes and reduced IgM levels. Our study suggests that rehabilitation programs with a focus on neuroplasticity and physical exercises in children with CP can reduce both cellular and humoral immune responses.

1. Introduction

Cerebral palsy (CP) is a lifelong physical disability as a consequence of upper motor neuron impairment, and it affects 1.5–2.5 children per 1000 live births with an estimated prevalence of 17 million people globally (Graham et al., 2016). CP imposes a huge financial burden on both parents and society. In the US, the estimated total lifetime cost per CP patient was $1.2 million in 2004, and approximately the same lifetime cost was reported in Denmark in 2009 (Tonmukayakul et al., 2018). CP manifests with features of movement disorders due to non-progressive brain lesions during the antenatal, perinatal, or early postnatal period. It is predominantly spastic motor forms of CP that are encountered in clinical practice, and the implications of spasticity include gait disturbances and fatigue (Reid et al., 2018). There are currently no indications that lifespan is affected in adults with mild to moderate CP (Peterson et al., 2012). Morbidity and even the mortality of children with CP are primarily due to recurrent and chronic respiratory infections (Proesmans, 2016; Seddon and Khan, 2003). The complex interplay between acknowledged risk factors such as aspiration, insufficient cough, upper airway obstruction, and progressive kyphoscoliosis heightens the risk of hospital admissions due to chest infection. Thus, chest physiotherapy is recommended to children with CP to reduce the severity of chest infection and to prevent the development of complications (The Royal Children's Hospital Melbourne, 2005). Very little is known about the extent to which the immune system is engaged in patients with CP, but it seems that these children carry genes that up-regulate the production of pro-inflammatory cytokines in response to various unfavorable factors such as intra-amniotic infection and preterm birth (Gibson et al., 2006). Moreover, Wu and Li (2015) found 2-fold increased levels of pro-inflammatory TNF-a in children with CP and suggested that inflammation may persist later in life reflecting the reconstruction of cerebral function after birth. There are also reports of increased IL-6 levels in patients with CP (Bi et al., 2014; Pingel et al., 2019).

Altered immune responses to environmental factors can also be caused by CP-associated regulatory polymorphism of the β-2 adrenergic receptor (Gibson et al., 2008) via several mechanisms, including C-reactive protein (CRP) secretion. This is supported by the study by Pingel et al. (2019) showing 8-fold higher levels of CRP in the plasma of

Abbreviations: CD3, cluster of differentiation 3; CP, cerebral palsy; GMFCS, Gross Motor Function Classification System for Cerebral Palsy; GMFM-88, Gross Motor Function Measure-88; CRP, C-reactive protein; IgG, immunoglobulin G; IL-6, interleukin 6; TNF-a, tumor necrosis factor alpha.

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children with CP compared to healthy adults. Moreover, an epigenetics study in newborn twins identified the activation of genes associated with inflammation and leukocyte-mediated immune responses causing the development of CP in the affected twin (Mohandas et al., 2018) thus reflecting the crosstalk between the innate and adaptive immune systems (Srivastava et al., 2019), i.e. lymphocytes are inappropriately activated in pathologies with prolonged inflammation.

Over the past decade, much research has been focused on the rehabilitation needs of children with CP with the intention to enhance physical performance and to minimize the aggravating factors, such as epilepsy and feeding challenges, in order to provide personalized care for this group of patients (Graham et al., 2016). The main approach to managing this motor disability is rehabilitation with a primary goal of reducing secondary musculoskeletal deformity rather than treating the primary central neurological deficit (İçağasıoğlu et al., 2015; Reid et al., 2010). The motor performance of CP patients is usually evaluated using the Gross Motor Function Measure-88 scale (GMFM-88), a recognized standardized test that has been used since 1993 and has been shown to be both a sensitive and reliable examination scale (Russell et al., 1993). Another major focus of research is pro-inflammatory dysregulation in infants with subsequent development of CP due to cytokine regulatory polymorphism, especially TNF-a (Gabriel et al., 2016; Gibson et al., 2006). A clinical study demonstrated the benefit of bone marrow mononuclear cell transplantation – i.e. a mixture of hematopoietic stem cells, mesenchymal stem cells, endothelial progenitor cells, macrophage, and lymphocytes – on the efficacy of rehabilitation in children with CP, and this effect was hypothesized to be through a reduction in inflammation (Sharma et al., 2015). In contrast, little is known about the effect of rehabilitation on the adaptive immune system in children with CP.

The aim of this study was to test children with CP for altered levels of TNF-a, IL-6, CRP, $CD22^+$ B-cells, and $CD3^+$ T-cells, with a focus on $CD4^+$ and $CD8^+$ T-cells. We found elevated levels of T-cells and B-cells, and we further analyzed the B-cell function via levels of different immunoglobulins. Another aim was to investigate the effect of rehabilitation on the immune system, and we found significant decreases in levels of IgA along with a trend for a decrease in B-lymphocyte numbers and IgM levels.

2. Material and methods

2.1. Subjects

A total of 23 children with the spastic form of CP were enrolled in the study group, and 15 healthy children (with a moderate level of physical activity) were enrolled in the control group. One subject was excluded from the study because of the parents' decision to withdraw before the completion of the improvement measurements; therefore, the second stage of the investigation only included samples from 22 subjects with CP. All subjects were native-born Ukrainians. Children with clinically confirmed spastic CP were recruited from the Sumy Regional Center for Social Rehabilitation of Children with Disabilities and the Department of Physical Education and Sports of Sumy State University, Ukraine. The spastic form of CP was diagnosed according to the definition, classification, and diagnostic criteria recommended by the guidelines of the Royal College of Physicians, London, Paediatric Stroke Working Group (Stroke in childhood - clinical guideline for diagnosis management and rehabilitation, 2004) and of the New York State Department of Health (Early Intervention Program - New York State Department of Health, 2006). Caregivers of children from both the control and study groups denied any medication intake by the children. Motor improvement was calculated as the GMFM-88 score before rehabilitation subtracted from the GMFM-88 score after rehabilitation in percentages (% gain in motor function = % GMFM-88 score after rehabilitation − % GMFM-88 score before rehabilitation). The severity of motor dysfunction was evaluated using the Gross Motor Function Classification System for Cerebral Palsy (GMFCS) (Gray et al., 2010).

The inclusion criteria were 1) a diagnosis of spastic CP, 2) age 1.5–10 years, and 3) stable health. Exclusion criteria were 1) a history of epilepsy or fever in the preceding two months, 2) inflammatory response syndrome, 3) diseases with a pro-inflammatory profile, including progressive amyotrophy, encephalomyelitis, severe malnutrition, myasthenia gravis, epilepsy, vision/hearing disorders, or other severe pediatric diseases, 4) other endocrine disorders, metabolic disorders, autoimmune diseases, or genetic diseases, and 5) clinical symptoms of acute and/or chronic infection. As required for studies involving human subjects, all caregivers of the participants completed a signed informed consent form prior to enrollment in the study. All experiments carried out in this study were approved by the ethical committees at Sumy State University and complied with the rules and regulations of the Ukrainian Ministry of Health and the Directive 2001/20/EC of the European Parliament and of the Council of 4 April 2001.

2.2. Sample collection and preparation

This study proceeded in three stages – specifically, motor performance assessment and blood sample analysis before rehabilitation, after 15 days of rehabilitation, and after 30 days of rehabilitation in the study group. Blood samples from the control group were collected once. The rehabilitation program for the CP patients was scheduled for 5 sessions per week (Saturdays and Sundays were days off) for 6 weeks (42 days in total). This rehabilitation duration was based on a 4-week training intervention, which resulted in leukocyte epigenetic reprogramming (Denham et al., 2015, 2016). There are several strategies to influence the immune system, such as nutritional interventions, vitamin and minerals intake, probiotics, and even pharmaceuticals (Mrityunjaya et al., 2020). In our study we chose the rehabilitation exercises program provided in the form of physical therapies based on the principles of neuroplasticity and muscle strengthening and stretching, i.e. task-specific skill training to improve motor coordination and performance along with physical training to address muscle weakness and decreased fitness. The essential advantage of the proposed method is a comprehensive scientific database of reduced associated adverse health outcomes in children with CP from inactive lifestyles (Graham et al., 2016).

During medical examination prior to blood collection, no pathological findings were observed. Children and their caregivers indicated no complaints, and body temperature values were within the normal range. Children and their caregivers reported no sleep disturbances prior to blood collection. Blood samples were collected from fasting subjects on Mondays between 8:45 and 9:10 in the morning to control for diurnal fluctuations in immunologic markers, and blood sampling was done at least 65 h after the last rehabilitation session (in case the last session was scheduled in the afternoon on Friday) in order to eliminate any immunomodulatory effects of acute exercise. The study was performed from November to March.

Heparinized blood for the examination of leukocytes, neutrophils, and lymphocytes was prepared at the time of subject enrollment. Serum was prepared by centrifugation at $1000\times g$ and stored at -20 °C until analysis for TNF-a, IL-6, CRP, IgM, IgG, and IgA.

2.3. Laboratory testing results

Leukocytes and the predominant subpopulation, i.e. neutrophils, were isolated by gradient centrifugation, and cells were counted in all squares in a Goryaev chamber under a microscope. All analyses were performed by a medical laboratory practitioner.

The direct antibody rosette assay was used for immunophenotyping lymphocytes according to the manufacturer's instructions for suspensions of erythrocytes coated with monoclonal antibodies from Granum (Ukraine). This is a well-established diagnostic method for immunologic evaluation in Ukraine and has been used in research by Duda and Kotsyubaylo (2016) and by Hruzevskyi et al. (2021). A sheep erythrocyte assay is used to quantify human T- and B-cells by measuring number of rosettes. Rosette is a lymphocyte (expressing specific CD) interacting with three or more sheep erythrocytes coated with monoclonal

antibodies (either anti-CD3, anti-CD4, anti-CD8 or anti-CD22). This is a modification of original sheep erythrocyte assay where T-cells were quantified by measuring number of rosettes created by interaction of CD58 (expressed on sheep erythrocyte) and CD2 (expressed on T-cells). Unlike monoclonal IgG - cluster of differentiation interaction, rosettes due to CD58 - CD2 interaction are temperature dependent, i.e. they disintegrate at 37 °C (Jondal et al., 1972). Thus, the incubation for 40 min at 37 °C allows to control for rosette formation due to CD58 - CD2 interaction. First, four suspensions of sheep erythrocyte coated with monoclonal antibodies against CD3, CD4, CD8, or CD22 were collected. Next, a leukocyte suspension was prepared. Blood was taken from a vein and collected in a test tube with 200–250 U/mL heparin. A total of 3 ml of blood was collected for each sample. Lymphocytes were separated from other blood cells by centrifugation at a gradient density of 1.077. Harvested cells (lymphocytes) were washed two or three times in saline with a pH of 7.2–7.4. In order to obtain a representative number of cells for quantification, the desired concentration was 20 cells in a large square of the Goryaev chamber. Suspensions of sheep erythrocytes were resuspended without foaming. A total of 50 μL of sheep erythrocytes coated with anti-CD3 monoclonal antibodies per sample were transferred to a sterile test tube, and 50 μL of lymphocytes were added. Similar procedure was performed for sheep erythrocytes coated with anti-CD4, anti-CD8, anti-CD22 monoclonal antibodies. The four mixtures were incubated for 40 min at 37 °C. Then centrifuged at 1850×*g* for 5 min and incubated again for 1 h at +4 °C to control for FcγR - IgG interaction (Passwell et al., 1978), i.e. it is blunted by incubation at 4 °C. The supernatants were removed and 50 μL of a 0.12% solution of glutaraldehyde was added to the precipitate, which was carefully resuspended to avoid foaming. The four mixtures were incubated for another 5–7 min and carefully resuspended. Next, the same procedure was performed for four mixtures: a smear of 1 cm^2 was swabbed on a glass coverslip. The slide was then dried and fixed with alcohol, and Romanowsky staining was applied. Using a light microscope with an immersion objective, the number of rosettes (lymphocytes, which bind three or more erythrocytes with CD-antibodies), was calculated along with the total number of lymphocyte subsets. Calculations were performed for $CD3^+$ T-cells, $CD4^+$ T-cells, $CD8^+$ T-cells and $CD22^+$ B-cells.

ELISA tests to quantify serum levels of TNF-a, IL-6, IgM, IgG, and IgA were performed according to the kits' instructions. The ELISA kits for TNF-a and IL-6 were purchased from Vector-Best (Russia), and the kits for IgM, IgG, and IgA were purchased from Xema (Russia). The ELISA tests were performed by medical practitioners at the Med-Online laboratory.

The serum CRP concentration was measured using a high sensitivity assay, i.e. turbidimetric latex agglutination method (CRPL3, Roche/Hitachi Cobas c-system 701/702, Roche Diagnostics GmbH, Mannheim, Germany).

2.4. Statistical analysis

Comparison of controls and children with CP by Fisher's exact test did not show a significant difference according to gender – the odds ratio was 0.88 (95% confidence interval −0.2870 to 0.3489) with the following presentation: 10 (43.48%) males and 13 (56.52%) females in children with CP and 7 (46.67%) males and 8 (53.33%) females in the control group; $P > .9999$. An unpaired *t*-test also showed no significant difference in age between the groups (6.42 ± 2.76 years in the controls and 4.91 ± 2.38 years in the children with CP; $P = .0816$). Fisher's exact test and the unpaired *t*-test were performed using GraphPad Prism version 8.1.2 (332). Two-sided testing was applied, and $P < .05$ was considered significant.

A prior sample size was calculated to justify the number of subjects needed for the investigation based on earlier research. Because Wu and Li (2015) reported that the plasma TNF-a level in the group of 54 CP subjects was 21.4 ± 6.2 pg/ml and in the group of 28 healthy controls was 11.2 ± 4.1 pg/ml, the present study would require a sample size of 6 for both groups to achieve a power of 80% and a level of significance of 5% (two sided). The sample size analysis was performed using G*Power software (version 3.1.9.4) (Faul et al., 2009). We increased the number of participants in the study to 23 children with CP in order to fulfill the requirements of the prior sample size calculations and to diminish the impact of heterogeneity in this clinical group.

The comparability of controls and children with CP in terms of immune system components was determined using a robust linear regression model designed using RStudio software (RStudio | Open source & professional software for data science teams, 2020) (Version 1.3.1073) with the MASS and repmod packages (rlm() and rob. pvals() functions) to account for outliers. The levels of immune system components (the level of TNF-a, IL-6, CRP; the number of leukocytes, neutrophils, $CD3^+$, $CD4^+$, and $CD8^+$ T-cells; the CD4+:CD8+ ratio; the number of $CD22^+$ B-cells; and the levels of IgM, IgA, and IgG) were chosen as the independent variables. The dependent variables in the model were group (controls, children with CP) and age. Considering the effect of sex on the levels of the components of immune system, we adjusted the values for both age and sex, but no differences were found (data not shown).

The efficacy of rehabilitation was explored using a linear mixed effect model (RStudio | Open source & professional software for data science teams, 2020) with the lme4 package (the lmer() function), and gain in motor function was the outcome variable. Age was used as the fixed factor, and severity of motor dysfunction (in terms of GMFCS) was added as a random intercept to account for variation between different severity levels of motor dysfunction.

A linear mixed effect model was designed using RStudio software (RStudio | Open source & professional software for data science teams, 2020) (Version 1.3.1073) with the lme4 and lmerTest packages (the lmer() function) to explore the effects of rehabilitation. Motor performance (GMFM-88 score) and levels of immune system components (the level of TNF-a, the number of leukocytes, the number of $CD22^+$ B-cells, the levels of IgM, IgA, IgG, and the numbers of $CD3^+$, $CD4^+$, and $CD8^+$ T-cells) were chosen as the outcome variables. The fixed factor in the model was time point (before rehabilitation, after 15 days of rehabilitation, and after 30 days of rehabilitation). Variation between individuals was accounted for in the model via 22 random intercepts associated with children with CP. Age values were mean-centered.

3. Results

3.1. Altered levels of immune system components

To better understand how the inflammatory status of CP compares to healthy children, we measured TNF-a levels in serum as a marker for systemic inflammation and found 3.80 pg/ml in healthy children vs. 11.15 pg/ml in children with CP ($P = .0358$). Adjustment of our data for age resulted in a loss of significance and a trend for 2-fold higher levels of TNF-a in children with CP (Fig. 1A, Table 1). Still, we found this way of interpretating our data to be more reasonable because it allowed us to compare groups of children with different age structures and to reproduce the results from a previous study (Wu and Li, 2015). We also found 1.5-fold higher levels of IL-6 in children with CP after adjusting for age (Table 1), which was similar to Pingel et al. (2019), although the differences were not significant in either study. In addition, CRP, a clinical marker for inflammation, did not differ significantly between children with CP and healthy children (Table 1). Values of CRP above 1 mg/L, which indicate low-grade systemic inflammation (Kamath et al., 2015), were registered in only 2 out of 23 patients with CP. Thus, the innate immune system was only modestly activated in children with CP.

We next assessed the influence of CP and associated ongoing inflammation on the adaptive immune system by measuring levels of B-cells, which are responsible for humoral immunity, and T-cells, which are involved in both humoral and cell-mediated immunity. The total leukocyte count was 17% ($P = .0179$) lower in the healthy controls (Fig. 1B), and to understand if such differences might be related to specific subpopulations of cells we analyzed the levels of dominating subpopulations

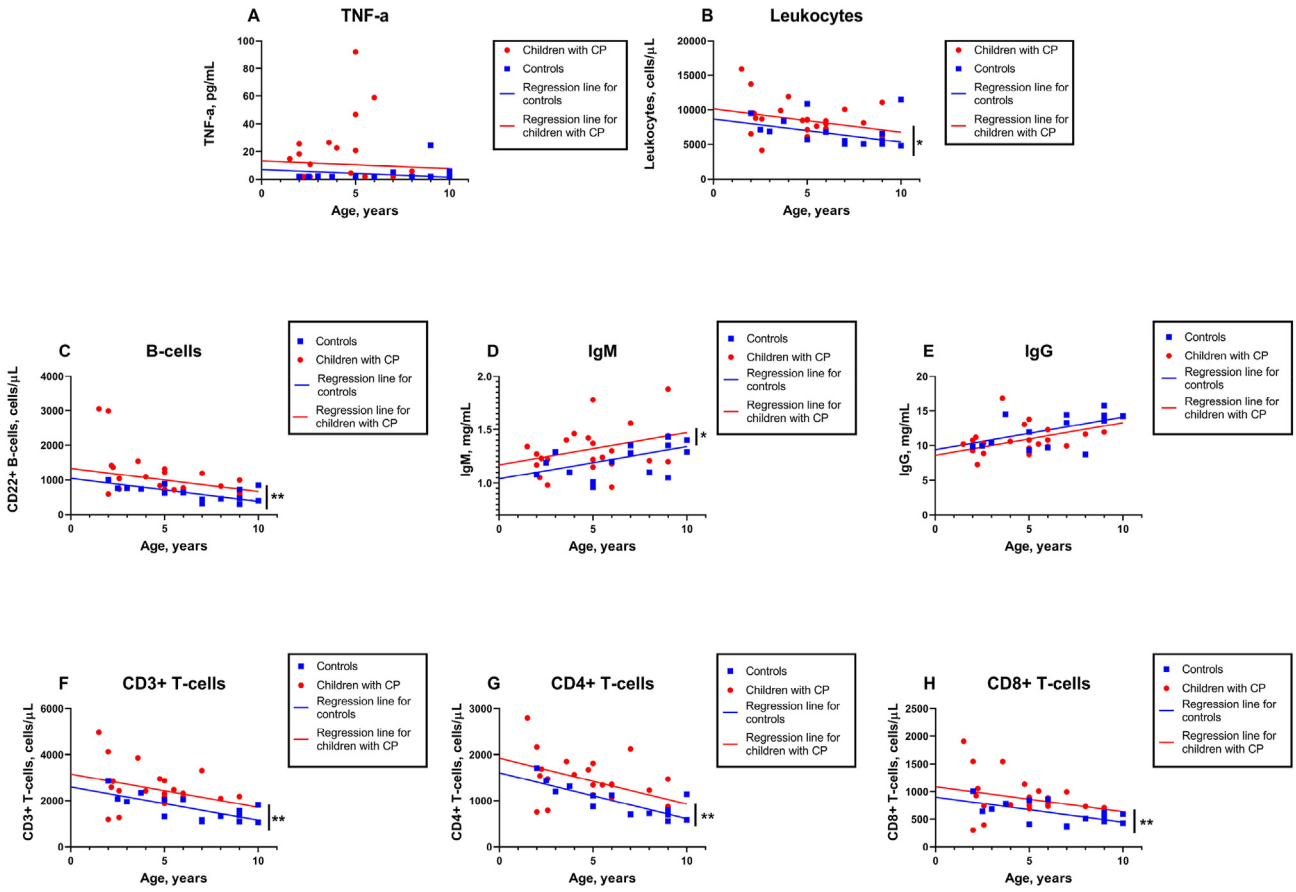

Fig. 1. Trends for increased (A) TNF-a levels and significantly increased numbers of (B) leukocytes, (C) $CD22^+$ B-cells, (D) IgM, (F) $CD3^+$ T-cells, (G) $CD4^+$ T-cells, and (H) $CD8^+$ T-cells were seen in children with CP (n = 23) in comparison to controls (n = 15). No significant difference in the level of (E) IgG was found in children with CP. Regression lines were calculated using results from robust linear regression rlm(). *P < .05, **P < .01.

Table 1
Immune characteristics of children with CP (n = 23) and healthy controls (n = 15) adjusted for age in a linear regression analysis.

Immune system components	Controls	Children with CP	P
TNF-a, pg/mL	7.09	13.31	.0613
IL-6, pg/mL	3.54	5.47	.1281
CRP, mg/L	0.02	0.03	.8761
Leukocytes, cells/μL	8694.43	10,135.61	.0179 *
Neutrophils, cells/μL	3450.02	3720.66	.3938
B-cells ($CD22^+$), cells/μL	1046.73	1335.35	.0040 **
IgM, mg/mL	1.04	1.17	.0378 *
IgG, mg/mL	9.39	8.57	.2181
IgA, mg/mL	2.04	2.00	.7214
$CD3^+$ T-cells, cells/μL	2601.68	3162.55	.0016 **
$CD4^+$ T-cells, cells/μL	1608.95	1926.34	.0035 **
$CD8^+$ T-cells, cells/μL	899.11	1088.46	.0095 **
CD4+:CD8+ ratio	1.86	1.87	.9601

The levels of immune system components correspond to the coefficient in the linear regression model, i.e. the predicted value of the independent variable; *P < .05; **P < .01.

of leukocytes, i.e. neutrophils and lymphocytes. The number of neutrophils was 8% higher in children with CP and did not differ significantly (P = .3938) (Table 1). Thus, we proceeded by immunophenotyping the lymphocyte subpopulations. B-cells were identified by the B-cell–specific CD22 molecule (Erickson et al., 1996; MoyrOn-QuirOz et al., 2002), which is expressed from early to late stages of maturation on all B-cell subsets in peripheral circulation but is gradually lost during the transformation from B-cells to memory B-cells and antibody-producing plasma cells (Dörner et al., 2015), thus allowing us to explore B-cells with a shorter lifespan (Jones et al., 2015). The total number of $CD22^+$ B-cells (Fig. 1C, Table 1) was 28% higher (P = .0040) in children with CP compared to healthy controls. Moreover, levels of IgM were higher by 13% (P = .0378) in the CP group (Fig. 1D, Table 1). However, immunoglobulins produced by plasma cells in a T-helper dependent manner (Stavnezer and Schrader, 2014), i.e. IgA and IgG (Fig. 1E), did not differ significantly (Table 1).

We next assessed the levels of T-cells by CD3 expression, and the total number of $CD3^+$ T-cells (Fig. 1F) was 22% higher (P = .0016) in children with CP. A similar increase of 20% was observed for $CD4^+$ T-cells (P = .0035) and an increase of 21% was seen for $CD8^+$ T-cells (P = .0095) (Fig. 1G and H) (Table 1) resulting in a CD4:CD8 ratio that was similar to that of healthy children (Table 1), and this allowed us to exclude viral infections and/or autoimmune reactions (Ahmad et al., 2013; McBride and Striker, 2017).

The significantly increased numbers of B-cells, and $CD4^+$ and $CD8^+$ T-cells without additional changes in the CD4+:CD8+ ratio suggested a common mechanism in the promotion of increased numbers of immune cells in children with CP.

Fig. 1. Immune changes in peripheral blood in children with CP.

3.2. Immunological effects of rehabilitation exercises

Motor disability in children with CP is associated predominantly with spastic transformation of skeletal muscles, which can be managed by rehabilitation approaches (Reid et al., 2010). To test if such rehabilitation might affect immune responses, we examined the locomotor performance of children with CP along with their immune status at the start and after 15 and 30 days of a rehabilitation program (42 days in total due

to additional weekends without rehabilitation exercises). First, we confirmed the efficacy of rehabilitation based on the principles of neuroplasticity, muscle strengthening, and stretching. The 30-day rehabilitation resulted in a gain in motor function for every child with CP with an average improvement of 6.73% when adjusted for age (a statistical process applied to rates of improvement that allowed us to compare children with CP of different ages). In addition, linear mixed effect regression analysis showed an effect of age on gain in motor function

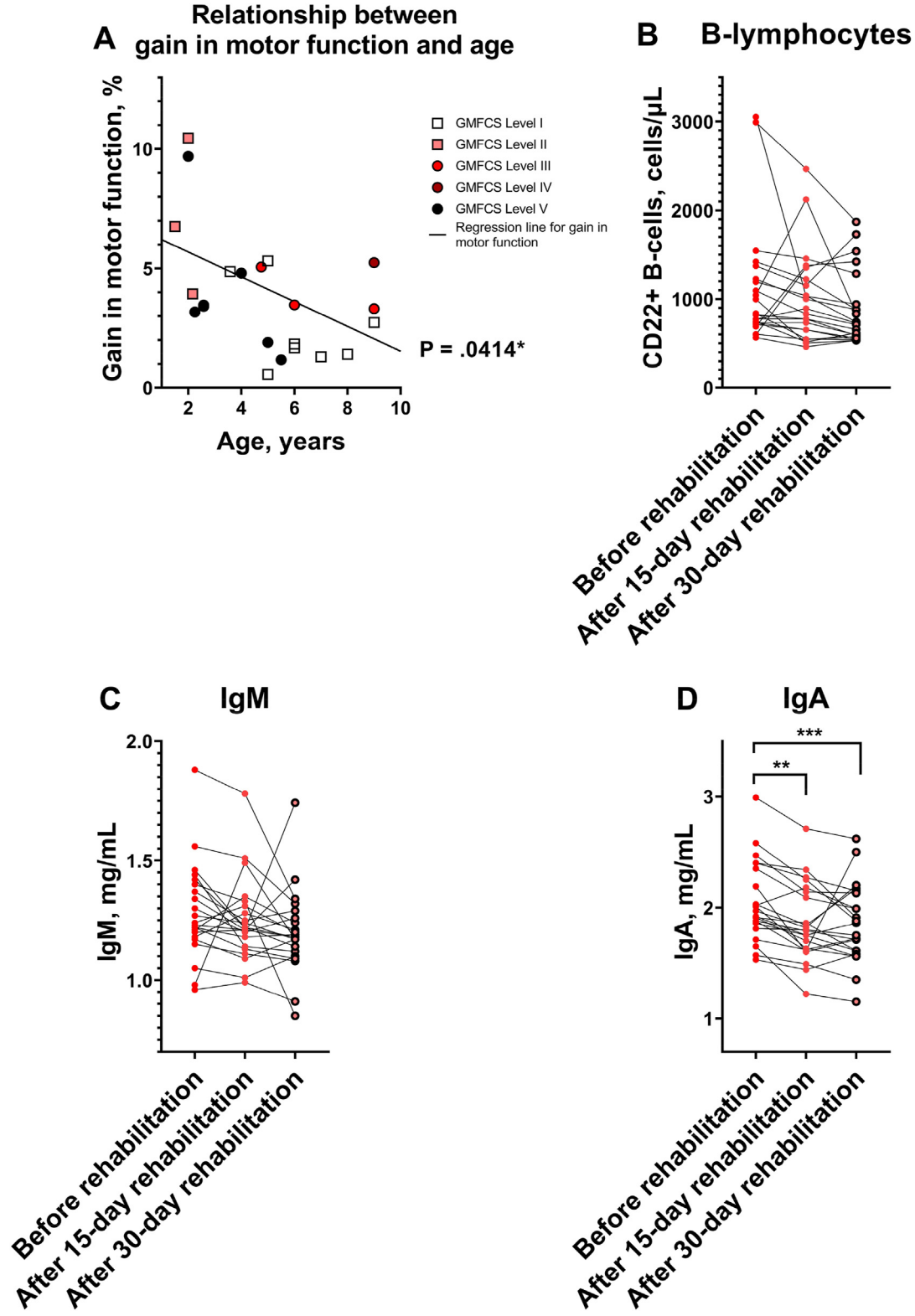

Fig. 2. (A) Relationship between gain in motor function and age in children with CP grouped according to severity of motor dysfunction (GMFCS) (the regression line was calculated using results from linear mixed effect regression analysis lmer()). (B) Trend for a decline in the number of $CD22^{+}$ B-cells in a direct rosette assay in peripheral blood of children with CP over the course of rehabilitation. (C) Trend for a decrease in IgM level in serum over the course of rehabilitation. (D) Decrease in IgA in serum over the course of rehabilitation; *P < .05, **P < .01, ***P < .001.

(P = .0414) (Fig. 2A), i.e. there was greater gain in motor function at earlier age. The complexity of the response to rehabilitation in this group of children was shown by the differences in vigor within the same age group, where motor improvement varied from one child to the next. These results are similar to the rehabilitation outcome after 30 days of intensive physical and occupational therapy for children with bilateral spastic CP seen in the study by Lee et al. (2015).

We next tested for pro-inflammatory TNF-a (Table 2), which displayed no significant difference after rehabilitation. This was in line with the study by Libardi et al. (2012) showing stable levels of TNF-a after 1 and even 16 weeks of resistance, endurance, and concurrent training in sedentary adults. In children with CP, we proceeded by analyzing leukocytes, which showed no significant changes. We next analyzed lymphocyte populations and their related molecules, and after 30 days of rehabilitation we observed a 21% decrease in the number of B-cells (the reduction did not reach significance due to interindividual variance in dynamics) (Fig. 2B). Notably, the levels of IgA decreased significantly by 10% (P = .0007) (Fig. 2D) with the same dynamics in almost all children. Complementary to this, the elevated IgM levels in children with CP showed a trend for a decrease of 6%, in contrast to stable IgG levels (Table 2). The different isotypes have different half-lives in serum, with IgG being the most stable isotype. It is, therefore, possible that changes in levels are not detected for IgG after 30 days. To verify that the change in levels was linked to the turnover rate of immunoglobulins, we followed changes in IgA and IgM by evaluating their levels three times with intervals of 15 rehabilitation days (Fig. 2D and C). Levels of IgA dropped by an average of 9% after 15 days of rehabilitation, while IgM showed only a 2% decrease after 15 days of rehabilitation and a 6% decrease after 30 days of rehabilitation, i.e. we observed rather stable levels followed by a rapid drop. However, no correlations were found between B-cells or IgA and IgM levels and motor improvement.

Fig. 2. Effect of motor improvement in children with CP due to rehabilitation on adaptive immune system components and correlation with age.

4. Discussion

In this study we investigated innate and adaptive immune system components in children with spastic CP compared to healthy age-matched children and the effect of rehabilitation on immune responses. Considering that muscle tissue composes up to 40% of the human body mass (Janssen et al., 2000), Peterson et al. (2012) speculated that spasticity causes a variety of metabolic disturbances in children with CP, including persistent obesity and sedentary behavior-induced inflammation. This hypothesis is supported by the results on elevated TNF-a levels in our study (Table 1) and by Wu and Li (2015). However, pro-inflammatory CRP levels were similar to those of the healthy controls. This might be partly explained by the average age of the participants, which was 5 years. Pingel et al. (2019) reported that children with CP (average age = 10 years) showed 7-fold higher CRP levels compared to adults with CP, although there was no significant difference between adults with CP and healthy adults. Thus, children might exhibit higher CRP levels that represent the beginning or the fading of subclinical infections that are more likely in younger age groups (Schlenz et al., 2014). Complementary to this, the relevance of age is supported by the study of Bi et al. (2014) that showed significantly increased IL-6 levels in children with CP under 2 years of age, whereas there was no significant difference in IL-6 levels between healthy adults and adults with CP (Pingel et al., 2019). In addition, Wu and Li (2015) reported 1.4-fold higher levels of TNF-a in children with CP under 4 years of age compared to older children with CP, which is in line with the negative slope when plotting TNF-a levels by age in our study (Fig. 1A). Further investigation is needed to verify whether decreases in pro-inflammatory cytokines in patients with CP are associated with the natural maturation of the blood-brain barrier (Moretti et al., 2015), as suggested by Wu and Li (2015) because both healthy adults and adults with CP exhibit similar levels of CRP and IL-6 (Pingel et al., 2019), or if they are associated with levels of sympathetic nervous system activity due to brain damage, as described by Rogers et al. (1998).

We decided to look further for correlation between cells and molecules of the adaptive immune system and spastic muscle function. Considering the reports by Denham et al. (2015, 2016) on epigenetic reprogramming of leukocytes by modified muscle activity, a molecular perspective might be of interest. Smith et al. (2009, 2012) studied wrist and hamstring muscles and reported distinct transcriptional profiles in spastic skeletal muscles. Of particular relevance is the reported increased expression of growth factors such as insulin-like growth factor 1 (IGF-1) and platelet-derived growth factor C (PDGFC) in spastic muscles (Smith et al., 2012). Serum IGF-1 has been reported to be associated with increased white blood cells, particular T-cells ($CD4^+$, $CD8^+$) and B-cells (Devi et al., 2015; Hinton et al., 1998), and PDGFC might mediate T-cell survival via PDGF regulation (Yang et al., 2010). Thus, induced growth factor stimulation of lymphocyte survival is consistent with our findings of enhanced levels of $CD3^+$ T-cells and both $CD4^+$ and $CD8^+$ T-cell subsets along with increased levels of $CD22^+$ B-cells.

To test the hypothesis that spastic muscle contractions affect the immune system, we evaluated the effect of rehabilitation, which is the generally accepted approach to managing spasticity (Shamsoddini et al., 2014), on adaptive immune system components. In this study we found that 30-day rehabilitation resulted in immune responses (Table 2) such as a reduction in both B-cells and IgA and IgM levels, but no reduction in T-cells. Of particular interest is the reduction in IgA levels (Fig. 2D) already after 15 days, whereas the reduction in IgM was slower but reached similar reduction after 30 days (Fig. 2C). This difference is in line with the mean half-life of immunoglobulins in serum – 2.6 days for IgA vs. 5 days for IgM and 13.8 days for IgG (Curtis and Bourne, 1973). The difference in serum stability led us to hypothesize that the observed decline in IgA and IgM was caused by a reduction in immunoglobulin production. In comparison, measurements of total $CD3^+$ T-cells along with $CD4^+$ and $CD8^+$ subsets showed steady levels of lymphocytes over the course of rehabilitation (Table 2). This is consistent with a 2-week--long investigation of peripheral blood T-cells in which the authors concluded that extended periods of physical activity, i.e. longer than 2 weeks, elicited significant changes in levels of T-cells (Brown et al., 2015). In contrast to T-cell dynamics, we observed a trend for a decline in levels of B-cells in children with CP towards those of healthy children after 30 days of rehabilitation (Fig. 2B). This is in line with the several-fold slower turnover rates – the percentage of a cell population replaced by new cells per day – of T-cells compared to B-cells (Westera et al., 2015) – where the median turnover rate in 42 days is 1.68% and 1.26% for naïve $CD4^+$ and $CD8^+$ T-cells, respectively, in comparison to

Table 2
Effect of rehabilitation in children with CP on the 15th and the 30th day.

Outcome variable	Before rehabilitation	After 15-day rehabilitation	After 30-day rehabilitation
GMFM-88 score, %	50.44	53.02***	54.33***
TNF-a, pg/mL	16.47	16.22	18.22
B-cells ($CD22^+$), cells/μL	1114.77	958.74	877.63
IgA, mg/mL	2.05	1.86**	1.85***
IgM, mg/mL	1.28	1.25	1.20
IgG, mg/mL	10.99	11.26	10.99
$CD3^+$ T-cells, cells/μL	2523.23	2741.41	2601.09
$CD4^+$ T-cells, cells/μL	1463.46	1578.68	1495.18
$CD8^+$ T-cells, cells/μL	886.59	992.27	954.14

The level corresponds to the coefficient in the linear mixed effect model, i.e. the predicted value of the outcome variable for the first fixed factor in the model. **P < .01; ***P < .001, when compared to the level before rehabilitation.

9.66% for naïve B-cells – thus allowing us to conclude that the faster turnover rate of the B-cells could have contributed to the trend for a decline. In addition, van Eyk et al. (2018) reported increased expression of CD24 and CD38 markers on B-cells in children with CP. These transitional markers are gradually lost during maturation and correspond to transitional B-cells (Bemark, 2015; Sanz et al., 2019), i.e. B-cells undergoing sequential differentiation stages from newly formed immature bone marrow B-cells to functionally competent B-cells, the lifespan of which is shorter compared to memory B-cells and plasma cells. Overall, our results allow us to hypothesize that the effect of rehabilitation on the adaptive immune system is due to the effect of diminishing the factors that regulate lymphocyte turnover, i.e. a reduction in anti-apoptotic factors results in decreased survival and a consequent reduction in the number of lymphocytes.

Motor improvement was negatively associated with age (Fig. 2A) and followed the decline in B-cells, IgA, and IgM. These results correspond to the age-related improvements reported by Lee et al. (2015) and support the general recommendations for early interventions (Hadders-Algra, 2014; Reid et al., 2015). Additionally, interesting patterns have been observed by comparing data on immune alterations within different time frames after brain damage and in relation to age. Rogers et al. (1998) showed increased B-cell levels in adults within short time periods after brain damage, although adults who had suffered from perinatal brain damage did not show significant differences compared to healthy controls. Thus, considering that the observed B-cell and IgA and IgM dynamics are rather a consequence of diverse molecular interactions, we propose looking at growth factors and how they are correlated with age (Meybosch et al., 2019) and are associated with molecular alterations in spastic muscle (Smith et al., 2012) in order to follow movement improvement and thus establish biomarkers for rehabilitation efficacy. Our study provides a basis for future research into the development of personalized rehabilitation strategies and the development of immunomodulation therapy, which can improve the quality of life of patients with CP and better integrate them into society.

The major limitation of the rehabilitation exercises program would be the duration needed to achieve noticeable motor improvement, which should be not less than a month, while in some cases it can take up to 6 months or even years. Another limitation is that children with CP are rehabilitated according to the severity of motor disability, i.e. children do different exercises. In addition, the duration of each exercise is also based on the individual capability of the child. In conclusion, there is a need for large groups children with the same severity of motor disability (nationwide level) to investigate the effect of precisely calculated muscle activity on the immune system. Another limitation of the program is rather questionable because on the one hand rehabilitation is considerably expensive (involving a lot of highly qualified medical professionals), while on the other hand rehabilitation is required on a regular basis in order to improve quality of life. Thus, rehabilitation can serve both the primary goal of improving motor activity and the secondary goal of improving immune status in children with CP.

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